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# AUTOCATALYTIC OXIDATION OF HEMOGLOBIN INDUCED BY NITRITE: ACTIVATION AND CHEMICAL INHIBITION

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**Abstract**—The nitrite ion is a direct causative agent for methemoglobinemia. Oxidation of hemoglobin to methemoglobin under aerobic conditions is induced by nitrite, catalyzed by methemoglobin in the presence of hydrogen peroxide, and inhibited by chemical reagents ranging from cysteine and ascorbic acid to sulfite. The stoichiometry of nitrate production is dependent on the initial $[NO_2^-]/[HbO_2]$ ratio, but reaches a limiting value of 1:1 $[NO_3^-]$: $[Hb^+]$ when $[NO_2^-]/[HbO_2] > 8$. Ascorbic acid is an exceptionally effective inhibitor for the autocatalytic oxidation, but its use does not affect the stoichiometry of nitrate formation. Sulfite reduces nitrate production to a level that is half that observed in its absence. These chemical inhibitors act upon the rapid autocatalytic stage for hemoglobin oxidation, but they do not influence the slow direct oxidation of hemoglobin by nitrite. The autocatalytic stage for hemoglobin oxidation results from nitrogen dioxide formed from nitrite through the peroxidase activity of methemoglobin. Peroxide and methemoglobin are formed during the initiation stage by electron transfer from nitrite that is kinetically first order in oxyhemoglobin and in nitrite.

**Keywords**—Methemoglobinemia, Nitrite, Oxyhemoglobin, Ascorbate, Peroxide, Nitrogen dioxide, Peroxidase activity, Autooxidation

## INTRODUCTION

The capability of sodium nitrite to oxidize hemoglobin ($HbO_2$) and related hemoproteins has been recognized for more than 100 years.[1] Lethal intoxications resulting in acute methemoglobinemia have been diagnosed, particularly in infants,[2,3] and fetal hemoglobin is especially sensitive to oxidation by nitrite.[4] Methemoglobinemia has been diagnosed in children affected by eating spinach containing excessive nitrite, and similar effects have been reported from the ingestion of fish and meat products containing a high concentration of nitrite.[5]

The principal toxic effect of the nitrite ion is oxidation of hemoglobin to methemoglobin ($Hb^+$), and considerable effort has been expended to identify the causative and preventive factors in this complex transformation.[6–9] Oxidation of hemoglobin under aerobic conditions occurs in two stages: a slow initial transformation that has been suggested to occur by single-electron transfer from nitrite to the bound dioxygen of hemoglobin[10] and a rapid subsequent autocatlytic oxidation that is promoted by the superoxide anion and peroxide.[11,12] The autocatalytic oxidation, which usually accounts for greater than 90% of the total oxidative conversion, is inhibited by catalase and by superoxide dismutase but at relatively high concentration.[11] As a preventive measure for nitrite oxidation of hemoglobin, ingestion of ascorbic acid[13] has been moderately effective, but the mechanism of its action remains unknown.

The $[HbO_2]:[NO_2^-]$ stoichiometry for nitrite induced oxidation of oxyhemoglobin was initially proposed to be 2:1.[14] A 2:3 stoichiometric ratio was advanced from investigations in which the minimum amount of nitrite required to convert oxyhemoglobin to methemoglobin was estimated by limiting the amount of nitrite employed.[6] Several years ago a 1:1 stoichiometry was proposed for this same reaction.[12] However, accurate nitrate determinations have not been performed in any previous study of nitrite induced hemoglobin oxidation.

Methemoglobin has been reported to have no effect on the conversion of oxyhemoglobin to methemoglobin by nitrite,[14] and this report has been generally accepted. More recently, however, the nitrite complex of methemoglobin was proposed to be essential for the au-

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tocatalytic stage of oxyhemoglobin oxidation.[8] Cyanide completely suppressed th autocatalytic oxidation, presumably through coordination with methemoglobin. As a result of the complexity of the nitrite-induced autocatalytic oxidation, mechanistic explanations for this transformation have disregarded a role for methemoglobin, and a variety of speculative transformations have been proposed.

We have recently proposed an explanation for the central events in the initiation and autocatalytic stages of hemoglobin oxidation by nitrite.[11] In addition to superoxide and peroxide, nitrogen dioxide and the peroxynitrate ion have been implicated. The involvement of peroxynitrate was proposed by analogy with the formation of peroxynitrite through the oxidative conversion of oxyhemoproteins to their corresponding ferrihemoproteins with nitric oxide.[15] Because chemical trapping of any one of these reaction intermediates would inhibit the autocatalytic stage of oxidation, chemical agents suspected to be effective as antioxidants have been intensively investigated. We find that certain chemical agents, of which ascorbate is the most notable, completely suppress autocatalytic oxidation, but that methemoglobin in combination with hydrogen peroxide activates this transformation.

## MATERIALS AND METHODS

Human hemoglobin A (type IV) and horseradish peroxidase (type VI) were obtained from Sigma Chemical Co. Hemoglobin was reduced with excess sodium dithionite and purified by passing the resulting aqueous solution through a G-25 Sephadex column using oxygen-saturated 0.05 M phosphate buffer at pH 7.0. Treatment of oxyhemoglobin at pH 7.8 with a 10-fold molar excess of iodoacetamide, relative to heme, afforded β-93 sulfhydryl-blocked hemoglobin.[16] Stock solutions of all reagents were prepared just prior to their use in oxygen-saturated 0.05 M phosphate buffer at pH 7.0 and 25.0°C, and they were maintained under an oxygen atmosphere.

Reactions were initiated with the injection of a concentrated nitrite solution into the oxyhemoglobin solution containing the chemical inhibitor. Time courses for hemoglobin oxidation were determined at 25.0°C by monitoring the decrease in absorbance at 576 nm with time using a Pye Unicam SP8-200 spectrophotometer. Control time courses for reactions employing only nitrite were performed both before and after inhibition experiments. Consistency in time courses for the autocatalytic stage was obtained only when identical reagent concentrations were employed. The use of hydrogen peroxide, even in concentrations less than 0.1 times that of hemoglobin monomer, enhanced the reproducibility of these time courses. Methemoglobin was the only spectrophotometrically visible product. Time courses for ascorbate and sulfite inhibited oxidations were fitted to an integrated single exponential process from which pseudo-first order rate constants were calculated. In each case, averaged values from a minimum of three kinetic determinations have been employed, and pseudo-first order kinetics were observed through at least two half-lives. The kinetic order with respect to nitrite concentration was determined from experiments using from 10- to 50-fold excesses of nitrite relative to heme.

Nitrate and nitrite with retention volumes of 9.3 ml and 5.7 ml, respectively, were cleanly separated from hemoprotein (retention volume <3.6 ml) on a Licrosorb-$NH_2$ reverse phase column with 50 mM potassium phosphate at pH 3.0 and analyzed at a detector wavelength of 210 nm.[17] Ascorbate and dehydroascorbate analyses employed the same column with a mobile phase of 50:50 acetonitrile:water containing 0.68 g/L of potassium phosphate and a detector wavelength of 254 nm.[18] Quantitative analyses were performed through reference to external standards.

## RESULTS

The accessible β-93 cysteine residues of hemoglobin have been previously recognized to inhibit the onset of autocatalytic oxidation of hemoglobin by nitrite.[19] The limited extent of this inhibition is described in Figure 1A by the time courses for nitrite ion oxidation of β-93 sulfhydryl-blocked and unmodified hemoglobin compared to the effect of added cysteine. Increased values for $t_{1/2}$, the time at which one-half of the reactant hemoglobin has been oxidized to methemoblogin, are observed with increased amounts of free cysteine, and the extent to which the β-93 hemoglobin cysteine residue inhibits autooxidation is seen to be comparable to that from added cysteine. This inhibition of the onset of the autocatalytic oxidation parallels the effect of superoxide dismutase and catalase[11] and is also observed with mercaptoethanol, as well as with either hydroxylamine or hydrazine. Time courses for nitrit - induced oxyhemoglobin oxidations with these chemical inhibitors resemble those reported in Figure 1A. The relative effectiveness of these inhibitors, determined from $t_{1/2}$ values, follows the order: L-cysteine < hydrazine < mercaptoethanol < hydroxylamine (Table 1). Although absolute values for $t_{1/2}$ in the presence of these inhibitors are subject to considerable variations (±20%), the relative inhibition effectiveness of these chemical agents is reproducible. Oxidized products of these chemical additives were detected by HPLC or mass spectral analyses following complete oxidation of

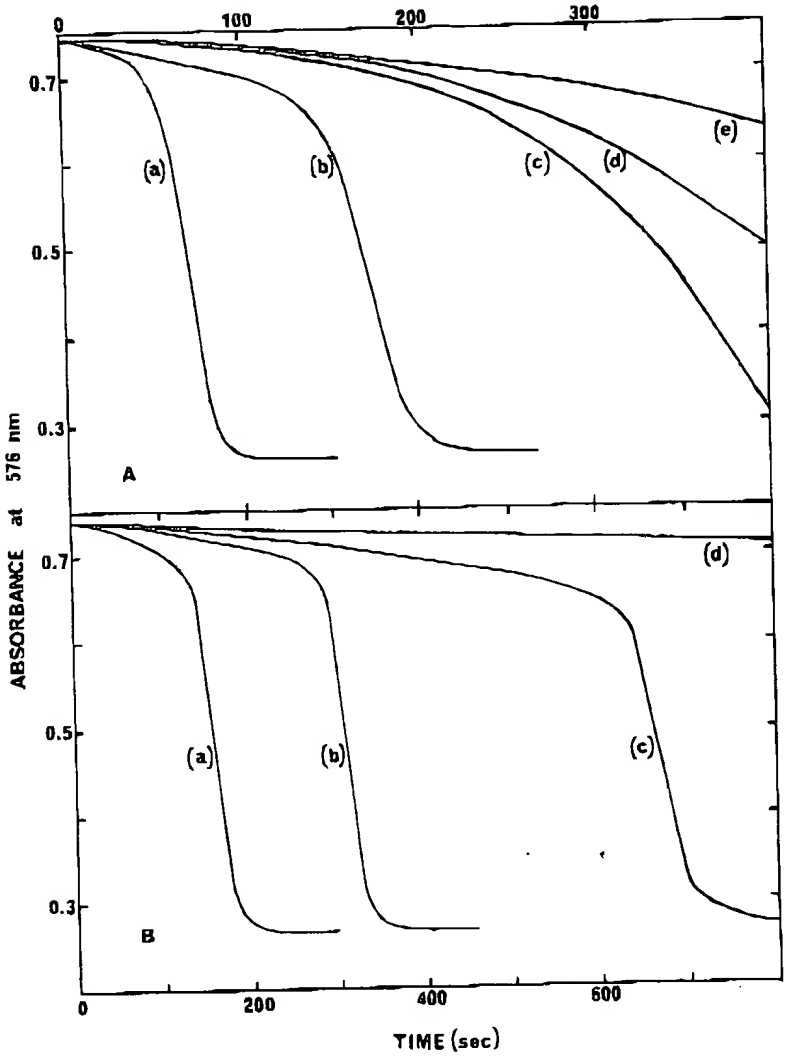


Fig. 1. Time courses for the nitrite ion induced oxidation of hemoglobin in oxygen-saturated 0.05 M phosphate buffer at 25.0°C, pH 7.0. A: Inhibition by the sulfhydryl group. $[HbO_2]$ = 48 μM, $[NaNO_2]$ = 960 μM; (*a*) β-93 sulfhydryl-blocked hemoglobin, (*b*) – (*e*) unmodified hemoglobin; [CysSH]/$[HbO_2]$ = 0.00 (*b*), 0.50 (*c*), 1.00 (*d*), 2.00 (*e*). B: Ascorbate inhibition. $[HbO_2]$ = 48 μM, $[NaNO_2]$ = 960 μM; $[AH_2]$/$[HbO_2]$ = 0.000 (*a*), 0.005 (*b*), 0.010 (c), 0.050 (*d*).

hemoglobin to methemoglobin, but their concentrations were not determined.

Ascorbic acid is reported to delay the onset of autooxidation following treatment of oxyhemoglobin with nitrite.[20,21] The cause of this inhibition has been attributed to reduction of methemoglobin, which is slow on the timescale for hemoglobin oxidation by nitrite.[22,23] Ascorbic acid alone oxidizes hemoglobin,[24] but its rate is sufficiently slow as to be negligible under the conditions employed in this study. We were therefore surprised to observe that ascorbic acid, even when present in molar quantities as low as $5 \times 10^{-3}$ times the concentration of heme, offered measurable inhibition of the autocatalytic transformation (Figure 1B). Unlike inhibition by cysteine, however, the time courses for autocatalytic oxidation are not significantly altered from that observed without added ascorbate, which suggests a special role for this antioxidant that may be catalytic. When ascorbic acid was used in concentrations equivalent to those of hemoglobin, the autocatalytic stage of oxidation was completely suppressed, and the rate of oxidation was observed under pseudo-first order kinetic conditions to be first order in both $[HbO_2]$ and $[NO_2^-]$ with an associated rate constant of 0.20 $M^{-1}\ s^{-1}$ at pH 7.0 and 25.0°C. By comparison, α-tocopherol, which is proposed to be an effective scavenger for superoxide generated within membranes,[25] is a relatively ineffective inhibitor of the autocatalytic stage of nitrite-in-

Table 1. Inhibition of nitrite-induced oxidation of oxyhemoglobin by chemical additives

| Chemical Additive | $t_{1/2}(+SH)/t_{1/2}(-SH)$* | Product |
|---|---|---|
| Cysteine | 2.2 | Cystine |
| $H_2NNH_2$ | 5.0 | $N_2$ |
| $HOCH_2CH_2SH$ | 9.7 | $(HOCH_2CH_2S)_2$ |
| Ascorbic acid | >100 | Dehydroascorbate |
| $Na_2SO_3$ | >100 | $Na_2SO_4$ |

Note. Using human hemoglobin (48 μM), $NaNO_2$ (96 μM), chemical additive (48 μM), pH 7.0, 0.05 M phosphate buffer, 25.0°C.
*Ratio of reaction half-time in the presence of an equivalent amount of the chemical additive (+SH), based on heme concentration, to the reaction half-time in its absence (−SH).

duced oxidation of hemoglobin. Only at concentrations equal to 10 times that of the hemoglobin monomer does α-tocopherol cause a doubling of the $t_{1/2}$ value in reactions performed under conditions identical to those reported in Table 1.

Ascorbic acid was oxidized to dehydroascorbate during hemoglobin oxidation by nitrite. Quantitative HPLC analyses of ascorbic acid ($AH_2$), dehydroascorbate (A), nitrite, and nitrate when $[NO_2^+]/[HbO_2] = 10$ indicated the stoichiometric relationship described in Eq. 1.

$$2HbO_2 + 2NO_2^- + AH_2 \longrightarrow 2Hb^+ + 2NO_3^- + A + 2OH^- \quad (1)$$

The 1:1 stoichiometry of nitrate production, relative to methemoglobin formation was reproducible over the range of ascorbate concentrations equal to half that of $[HbO_2]$ to four times that of $[HbO_2]$. Ascorbic acid was detected in all reaction mixtures in which its concentration exceeded one-half that of $HbO_2$. However, oxidation of $AH_2$ by molecular oxygen[26] may have occurred during this slow transformation, so that the actual effective stoichiometry of $AH_2$ for complete suppression of the autocatalytic oxidation may be less than that revealed in Eq. 1.

Hemoglobin oxidations by nitrite in the absence of ascorbic acid result in the production of nitrate at concentrations dependent on the initial $[NO_2^-]$ $[HbO_2]$ (Fig. 2A), except at $[NO_2^-]/[HbO_2] \geq 8$. With only a twofold excess of nitrite over that of $HbO_2$, nitrate is only a minor product in this oxidative transformation. At a $[NO_2^-]/[HbO_2]$ ratio of approximately 5, a steep rise in nitrate production occurs which approaches the limiting 1:1 stoichiometry. Complimentary studies in which the nitrite concentration was maintained constant at 940 μM showed no deviation from this limiting stoichiometry. With added peroxide, nitrate production increases with a unit correspondence to the amount of peroxide employed. Separate experiments demonstrated that methemoglobin catalyzes the otherwise unresponsive peroxide oxidation of nitrite to nitrate with an exact 1:1 $[H_2O_2]:[HO_2^-]$ stoichiometry. By itself, the hydrogen peroxide oxidation of $HbO_2$ is slow, characterized by a second-order rate constant of 8.6 $M^{-1}$ $s^{-1}$ at 25.0°C and pH 7.0.

Methemoglobin also catalyzes the autocatalytic oxidation of hemoglobin, but only when peroxide is combined with $Hb^+$ prior to addition to the reaction medium (Fig. 3A). When added to the $NO_2^-/HbO_2$ combination without initial peroxide, methemoglobin has no noticeable effect on the time course of the reaction that enters the autocatalytic stage of oxidation after a rel-

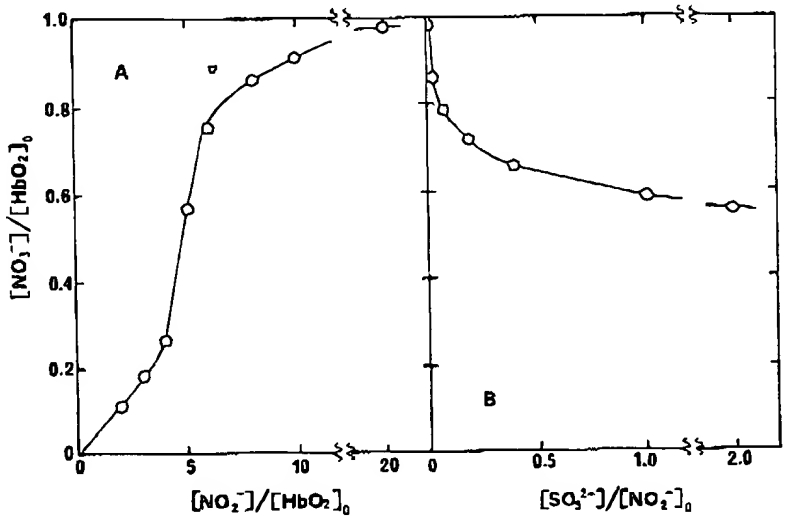


Fig. 2. Dependence of nitrate production on initial nitrite concentration (A) and on the sulfite to nitrite concentration ratio (B) for nitrite ion induced oxidation of sulfhydryl-blocked hemoglobin in oxygen-saturated 0.05 M phosphate buffer at 25°C, pH 7.0; $[HbO_2]$ = 47.8 μM. B: $[NaNO_2]$ = 940 μM.

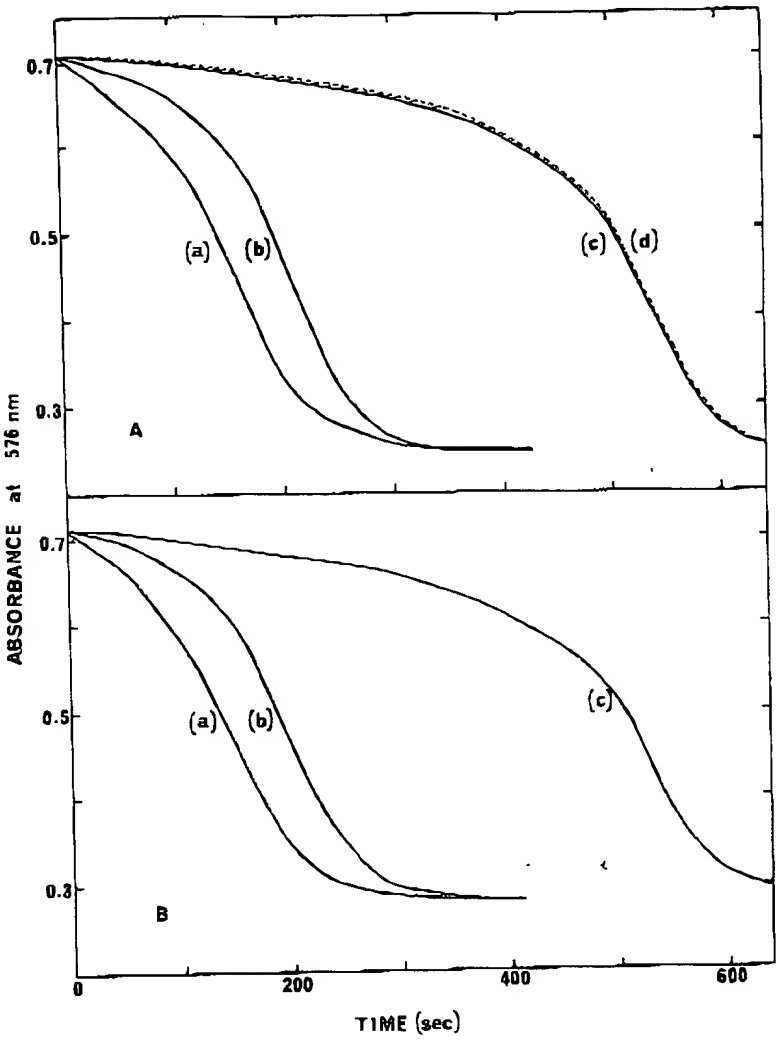


Fig. 3. Time courses for nitrite ion induced oxidation of sulfhydryl-blocked hemoglobin in oxygen-saturated 0.05 M phosphate buffer at 25.0°C, pH 7.0. A: $[HbO_2] = 47.3$ μM, $[NaNO_2] = 376$ μM; (*a*) $[Hb^+] = [HOOH] = 1.41$ μM; (*b*) $[Hb^+] = 0$ μM, $[HOOH] = 1.41$ μM; (*c*) $[Hb^+] = [HOOH] = 0$ μM; (*d*) $[Hb^+] = 1.41$ μM. B: $[HbO_2] = 47.0$ μM, $[NaNO_2] = 376$ μM; (*a*) [HRP] = 2.4 units, [HOOH] = 1.4 μM; (*b*) [HRP] = 0 units, [HOOH] = 1.41 μM; (*c*) [HRP] = 0 units, [HOOH] = 0 μM. Horseradish peroxidase alone inhibits the nitrite ion induced oxidation of $HbO_2$.

atively long induction period. Added peroxide reduces the time for this initial slow oxidation, but an induction period is still observed. When the combination of $Hb^+$ and peroxide is added, however, the induction period is eliminated, and the reaction immediately falls into the autocatalytic stage of oxidation. This same activity is reproduced when a catalytic amount of horseradish peroxidase/$H_2O_2$ is employed in place of $Hb^+/H_2O_2$ (Fig. 3B). Without added peroxide, horseradish peroxide is an effective inhibitor of the autocatalytic oxidation.

The addition of gaseous nitrogen dioxide also accelerated the onset of the autocatalytic reaction. Reactions performed under conditions identical with those described in Figure 1 using $[NO_2]/[HbO_2] = 1.0$, but without added peroxide or chemical inhibitors, resulted in rapid hemoglobin oxidation ($t_{1/2} < 60$ s compared to $t_{1/2} > 150$ s without added nitrogen dioxide) without evidence of an appreciable initial slow oxidation. In the absence of nitrite, the time course for oxidation of hemoglobin by nitrogen dioxide resembles that observed in nitrite oxidations with initial slow and subsequent rapid autocatalytic stages,[13] but the onset of autocatalytic oxidation is nearly 10 times faster than that observed using an equivalent concentration of nitrite and low concentrations of peroxide ($[H_2O_2]/[HbO_2] < 0.05$) do not alter the time courses for oxidation as is observed in nitrite oxidations.[11] However, the rate constant for dimerization of nitrogen dioxide to dinitrogen tetroxide in water is $4.8 \times 10^8$ $M^{-1}$ $s^{-1}$

at 20°C, and the equilibrium constant for dissociation of $N_2O_4$ is $1.53 \times 10^{-5}$ M under these same conditions.[27] Thus, even with the low concentrations of nitrogen dioxide that are employed, dinitrogen tetroxide is the principal nitrogen oxide species in solutions prior to its hydrolysis to nitrate and nitrite. Consequently, the acceleration of oxidation that we observe cannot be conclusively attributed solely to nitrogen dioxide, although the suggestion of its activity remains.

Oxygen atom acceptors such as dimethylsulfoxide or trimethylphosphite that were anticipated to be competitive with the nitrite ion for chemical entrapment of the peroxynitrate ion did not inhibit the oxidation of hemoglobin even when employed at 100-fold molar excess relative to heme. The time courses for these reactions were identical with those performed in the absence of these chemical agents. However, use of sodium sulfite not only inhibited the autocatalytic oxidation of hemoglobin by nitrite, but also caused a reduction in nitrate formation to a level that was only one-half that obtained in the absence of sulfite (Fig. 2B). Sulfate analyses were performed by gravimetric determination of barium sulfate. That these effects were not due to sulfite-induced oxidation of hemoglobin or to processes that interfere with the oxidation of $HbO_2$ by nitrite could be seen from the rate constant for oxidation at 25°C in the presence of excess sulfite, $k_{obs} = 0.091\ M^{-1}\ s^{-1}$, which was precisely one-half the rate constant obtained for hemoglobin oxidation in the presence of ascorbate.

## DISCUSSION

Our investigations of the oxidation of oxyhemoglobin by nitrite have employed nitrite concentrations in excess of the total heme concentration in order to determine the factors that control the onset of the autocatalytic stage of oxidation. In those few studies where $[NO_2^-]/[HbO_2] = 1$,[19] the initial and autocatalytic stages are mixed. Furthermore, time courses for nitrite oxidations of hemoglobin are notoriously variable,[11] particularly at low $[NO_2^-]/[HbO_2]$, and we have employed only those results that were reproducible through several sets of experiments.

In the initial stage of hemoglobin oxidation, nitrite undergoes electron transfer with $HbO_2$ that is best explained by the previously proposed mechanism,[10] but with the alteration that free peroxide exists in equilibrium with the iron-bound form rather than simply dissociated to methemoglobin and free peroxide:

$$HbO_2 + NO_2^- \longrightarrow HbO_2^- + NO_2 \quad (\text{or } Hb^+ + O_2^{2-} + NO_2) \qquad (2)$$

The ability of nitrite to react in this manner differentiates this anion from those that produce superoxide in anion-promoted autooxidations.[28] Nitrogen dioxide undergoes rapid dimerization to $N_2O_4$,[27] and subsequent hydrolysis results in the production of nitrite and nitrate (Eq. 3).

$$2NO_2 \rightleftharpoons N_2O_4 \xrightarrow{H_2O} NO_3^- + NO_2^- + 2H^+ \qquad (3)$$

As a consequence, total inhibition of the autocatalytic oxidation would be expected to result in the net production of one-half equivalent of nitrate based on oxidized hemoglobin (Eq. 4). The fate of peroxide is

$$2H^+ + H_2O + 2HbO_2 + NO_2^- \longrightarrow 2Hb^+ + NO_3^- + 2HOOH \qquad (4)$$

determined by the chemical methodology employed to inhibit the autocatalytic oxidation, and with sulfite inhibition the net stoichiometry is that represented by equation 5.

$$2H^+ + 2HbO_2 + NO_2^- + 2SO_3^{2-} \longrightarrow 2Hb^+ + NO_3^- + 2SO_4^{2-} + H_2O \qquad (5)$$

In the autocatalytic stage for oxidation of hemoglobin, nitrate is produced together with methemoglobin in a 1:1 stoichiometry when $[NO_2^-]/[HbO_2] > 8$. This same stoichiometry is observed in the presence of sufficient ascorbic acid to completely inhibit the autocatalytic transformation. However, at $[NO_2^-]/[HbO_2] < 8$, lower $[NO_3^-]/[Hb^+]$ product ratios are characteristic (Fig. 2A), and this observation presents a substantial limitation on the mechanistic development of the nitrite induced oxidation of oxyhemoglobin.

Methemoglobin-peroxide catalysis of the oxidation of nitrite to nitrate by peroxide provides a suitable starting point for understanding the complex mechanism for nitrite ion involvement in the oxidation of $HbO_2$. This catalysis is consistent with the following reactions (6–9):

$$Hb^+ + HOOH \longrightarrow HbO_2H + H^+ \qquad (6)$$

$$H^+ + HbO_2H + NO_2^- \longrightarrow [HbOH]^+ + NO_2 + OH^- \qquad (7)$$

$$H^+ + [HbOH]^+ + NO_2^- \longrightarrow Hb^+ + NO_2 + H_2O \qquad (8)$$

$$H_2O + 2NO_2 \longrightarrow NO_3^- + NO_2^- + 2H^+ \qquad (9)$$

whose sum is the net reaction of equation 10,

$$HOOH + NO_2^- \longrightarrow NO_3^- + H_2O \quad (10)$$

The involvement of $[HbOH]^+$, formally a hydroxyl-bound iron(IV) hemoglobin, is consistent with peroxidase activity,[29] and the mechanism for peroxide oxidation of nitrite that is presented in equations 6 through 9 is analogous to that proposed[30] for horseradish peroxidase catalysis of equation 10. In addition, this mechanism explains the limited extent of peroxide production in nitrite reactions with oxyhemoglobin.[31] By itself, however, the mechanism represented by equations 6 through 9 explains neither autocatalysis nor the variable stoichiometry of nitrite induced oxidations of oxyhemoglobin.

We previously proposed that formation of peroxynitrate by the reaction of nitrogen dioxide with $HbO_2$

$$HbO_2 + NO_2 \longrightarrow Hb^+ + O_2NOO^- \quad (11)$$

(Eq. 11) and its subsequent decomposition could account for the autocatalytic stage of nitrite induced hemoglobin oxidations.[11] Peroxide was accorded a role in the decomposition of $O_2NOO^-$, but this interpretation is inconsistent with the observed stoichiometry. However, decomposition of the unstable peroxynitrate ion in aqueous solution has been investigated,[32] and reaction 12 was found to be

$$O_2NOO^- \longrightarrow NO_2^- + O_2 \quad (12)$$

the dominant pathway above pH 5. An alternate pathway involving decomposition to nitrogen dioxide and superoxide could not be excluded from consideration, and this mode of decomposition could explain the inhibition of the autocatalytic oxidation by superoxide dismutase.[11,12] Thus, if equations 6, 7, and 8, and two times 11 and 12 represent the sole fate of peroxide and nitrogen dioxide, the net transformation of equation 13 does

$$HOO^- + 2HbO_2 + 3H^+ \longrightarrow 2Hb^+ + 2O_2 + 2H_2O \quad (13)$$

not involve nitrate production even though nitrogen dioxide is intimately involved in the overall process. However, this transformation does not explain autocatalysis in the oxidative transformation.

Autocatalysis in the nitrite induced oxidation of $HbO_2$ requires that equation 12 cannot be the sole fate of peroxynitrate in solution. Indeed, this relatively unexplored peroxide species is known to undergo electron transfer reactions[33] which, if directed to $HbO_2$, accounts for autocatalysis (Eq. 14 or 15). In the former case, equations 11 and 14 describe

$$2H^+ + O_2NOO^- + HbO_2 \longrightarrow Hb^+ + HOOH + NO_2 + O_2 \quad (14)$$

$$HbO_2 + O_2NOO^- \longrightarrow Hb^+ + O_2^- + NO_2^- + O_2 \quad (15)$$

a net transformation (Eq. 16) which accounts for autocatalysis,

$$2H^+ + 2HbO_2 \longrightarrow 2Hb^+ + HOOH + O_2 \quad (16)$$

catalysis by peroxide, and the observed limiting 1 : 1 $[NO_3^-]:[Hb^+]$ stoichiometry when $[NO_2^-]/[HbO_2] > 8$ and equation 2 is incorporated into this scheme. Equation 15 is consistent with the results of trapping experiments using superoxide dismutase,[11,12] and this transformation accounts for the production of a paramagnetic species whose free radical center is not nitrogen during the autocatalytic stage of oxidation.[6]

These mechanistic analyses for peroxynitrate involvement in the nitrite induced oxidation of oxyhemoglobin explain the extremes in nitrate production (Figure 2A). However, the complexity of the transformation does not allow us to exclude from consideration reactions in which peroxynitrate serves as a surrogate for peroxide in equations 6 and 7 or in which nitrogen dioxide undergoes dimerization and hydrolysis (Eq. 3), nor is it possible to provide kinetic justification for either limiting mechanism other than by a qualitative description of the autocatalytic stage for oxyhemoglobin oxidation. Rate constants for fundamental steps in this mechanistic analysis are required for further elaboration of this transformation.

The action of chemical inhibitors, except sulfite, on the nitrite induced oxidation is consistent with equations 17 and 18:

$$H^+ + HbO_2H + SH \longrightarrow [HbOH]^+ + H_2O + S \quad (17)$$

$$[HbOH]^+ + SH \longrightarrow Hb^+ + H_2O + S \quad (18)$$

where SH is nitrite or may be any of the series of chemical inhibitors including ascorbic acid and cysteine. The $[NO_3^-]:[Hb^+]$ stoichiometry for ascorbate inhibition of the autooxidation and the associated rate constant for oxidation, which is twice that for sulfite inhibition, suggests that ascorbate does not interfere with reaction 11.

In the presence of hydrogen peroxide, equation 6 is

operative and the catalytic role of methemoglobin combined with hydrogen peroxide is defined. That cyanide can completely suppress the autocatalytic stage of oxidation is consistent with the competition between cyanide and peroxide for association with methemoglobin. Methemoglobin added to the reaction solution without hydrogen peroxide is ineffective as a catalyst because of nitrite coordination to methemoglobin. Inhibition of the nitrite induced oxidation of $HbO_2$ by horseradish peroxidase employed without initial peroxide is not apparent at this time, but catalysis of the autocatalytic stage for oxidation by horseradish peroxidase with precisely the same time course as for methemoglobin catalysis (Figure 3) substantiates the peroxidase activity of methemoglobin in the nitrite induced oxidation of hemoglobin.

Although each inhibitor affects the overall transformation according to its particular chemistry, the influence of sulfite on the overall reaction stoichiometry is worthy of separate consideration. Horseradish peroxidase catalysis of the peroxide oxidation of sulfite is known to occur without intermediate formation of Compound II, formally analogous to $[HbOH]^+$, whereas the oxidation of nitrite occurs in two sequential steps.[30] Thus, sulfite is capable of intercepting $HbO_2H$ without generating $[HbOH]^+$ and, with this inhibitor, nitrate probably results solely from hydrolysis of the nitrogen dioxide dimer generated according to reaction 3.

Results that we have obtained suggest a complex role for peroxide and nitrogen dioxide in nitrite induced methemoglobinemia. The initiation stage is now well defined by electron transfer between nitrite and oxyhemoglobin resulting in the production of nitrogen dioxide and the methemoglobin-peroxide complex. Nitrogen dioxide undergoes subsequent rapid oxidation of hemoglobin, and the resulting peroxynitrate is also capable of electron transfer with hemoglobin which accounts for the autocatalytic stage of oxidation. However, dimerization of nitrogen dioxide with subsequent hydrolysis and dissociation of peroxynitrate to nitrite and oxygen compete with electron transfer in this complex transformation. In addition, the methemoglobin-peroxide complex catalyzes the oxidation of nitrite to nitrate, presumably through nitrogen dioxide.

Nitrogen dioxide is reported to cause methemoglobinemia, but its role in respiratory disorders has received considerably greater attention.[34–36] The present investigation suggests that nitrogen dioxide is a direct oxidant of hemoglobin under aerobic conditions and that its ability to cause formation of methemoglobin is comparable to that of nitrite. Unlike nitrite, however, peroxide does not affect the onset of autocatalytic oxidation unless or until nitrite is formed from nitrogen dioxide through hydrolysis of dinitrogen tetroxide or dissociation of oxygen from peroxynitrate.

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